PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
B01D 15/00, 15/08, C12Q 1/68, C08F 112/08, C07H 19/00

A1

(11) International Publication Number: **WO 98/48913**

(43) International Publication Date: 5 November 1998 (05.11.98)

(21) International Application Number: PCT/US98/08388

(22) International Filing Date: 23 April 1998 (23.04.98)

(30) Priority Data:

60/044,856	25 April 1997 (25.04.97)	US
60/059,636	23 September 1997 (23.09.97)	US
60/062,321	17 October 1997 (17.10.97)	US
60/063,628	27 October 1997 (27.10.97)	US
60/067,679	5 December 1997 (05.12.97)	US
60/077,875	13 March 1998 (13.03.98)	US
09/058,580	10 April 1998 (10.04.98)	US

(71) Applicant *(for all designated States except US)*: TRANSGENOMIC, INC. [US/US]; 2032 Concourse Drive, San Jose, CA 95110 (US).

(72) Inventors; and

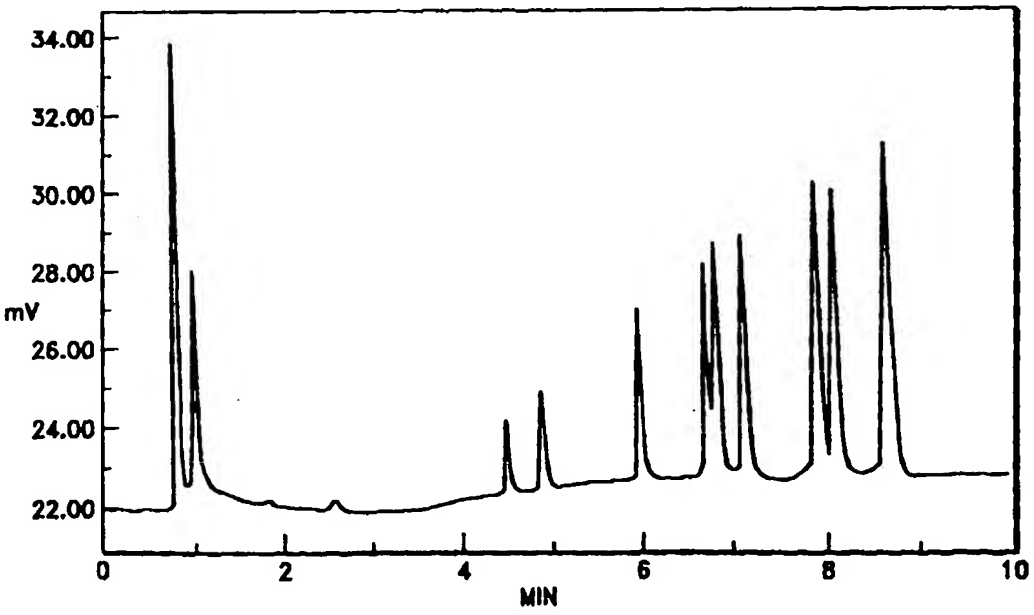
(75) Inventors/Applicants *(for US only)*: GJERDE, Douglas, T. [US/US]; 12995 Woodside Drive, Saratoga, CA 95070 (US). HAEFELE, Robert, M. [US/US]; (US). TAYLOR, Paul, D. [US/US]; 248 Hawthorne Avenue, Palo Alto, CA 94301 (US).

(74) Agent: WALKER, William, B.; Enterprise Law Group, Inc., Suite 280, 4400 Bohannon Drive, Menlo Park, CA 94025 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: POLYNUCLEOTIDE SEPARATIONS ON NONPOROUS POLYMER BEADS



(57) Abstract

The present invention describes a chromatographic method for separating polynucleotides with improved separation and efficiency. The method uses nonporous polymeric beads having a non–reactive, non–polar surface, and made from a variety of different polymerizable monomers.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TITLE OF THE INVENTION

POLYNUCLEOTIDE SEPARATIONS ON NONPOROUS POLYMER BEADS

FIELD OF THE INVENTION

The present invention is directed to the separation of polynucleotides using nonporous polymeric beads.

BACKGROUND OF THE INVENTION

Separations of polynucleotides such as DNA have been traditionally performed using slab gel electrophoresis or capillary electrophoresis. However, liquid chromatographic separations of polynucleotides are becoming more important because of the ability to automate the analysis and to collect fractions after they have been separated. Therefore, columns for polynucleotide separation by liquid chromatography (LC) are becoming more important.

High quality materials for double stranded DNA separations previously have been based on polymeric substrates disclosed in U.S. Patent No. 5,585,236, to Bonn et al., which showed that double-stranded DNA can be separated on the basis of size with selectivity and performance similar to gel electrophoresis using a process characterized as reverse phase ion pairing chromatography (RPIPC). However, the chromatographic material described was limited to nonporous beads substituted with alkyl groups having at least 3 carbons because Bonn et al. were unsuccessful in obtaining separations using polymer beads lacking this substitution. Additionally, the polymer beads were limited to a small group of vinyl aromatic monomers, and Bonn et al. were unable to effect double stranded DNA separations with other materials.

A need continues to exist for chromatographic methods for separating polynucleotides with improved separation efficiency and resolution.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a chromatographic method for separating polynucleotides with improved separation and efficiency.

Another object of the present invention is to provide a method for separating polynucleotides using nonporous polymer beads having a non-reactive, non-polar surface.

It is another object of this invention to provide the chromatographic separation of polynucleotides using nonporous polymeric beads made from a variety of different polymerizable monomers.

It is a further object of this invention to provide the chromatographic separation of polynucleotides using polymeric beads which can be unsubstituted, methyl-substituted, ethyl-substituted, hydrocarbon-substituted, or hydrocarbon polymer-substituted.

Yet another object of the present invention is to provide improved polymer beads by including steps to remove contamination occurring during the manufacturing process.

Still another object of the invention is to provide a method for separating polynucleotides using a variety of different solvent systems.

These and other objects which will become apparent from the following specification have been achieved by the present invention.

In summary, the method of this invention for separating a mixture of polynucleotides comprises flowing a mixture of polynucleotides having up to

1500 base pairs through a separation column containing beads having an average diameter of 0.5 to 100 microns, and separating the mixture of polynucleotides using Matched Ion Polynucleotide Chromatography (as defined hereinbelow). The beads are preferably made from polymers, including mono- and di-vinyl substituted aromatic compounds such as styrene, substituted styrenes, alpha-substituted styrenes and divinylbenzene; acrylates and methacrylates; polyolefins such as polypropylene and polyethylene; polyesters; polyurethanes; polyamides; polycarbonates; and substituted polymers including fluorosubstituted ethylenes commonly known under the trademark TEFLON. The base polymer can also be mixtures of polymers, non-limiting examples of which include poly(styrene-divinylbenzene) and poly(ethylvinylbenzene-divinylbenzene). The polymer can be unsubstituted, or substituted with a moiety selected from the group consisting of methyl, ethyl, hydrocarbon, or hydrocarbon polymer. The hydrocarbon polymer optionally has from 23 to 1,000,000 carbons. The beads preferably have an average diameter of about 1 - 5 microns. In the preferred embodiment, precautions are taken during the production of the beads so that they are substantially free of multivalent cation contaminants and the beads are treated, for example by an acid wash treatment, to remove any residual surface metal contaminants. The beads of the invention are characterized by having a DNA Separation Factor (as defined hereinbelow) of at least 0.05. In a preferred embodiment, the beads are characterized by having a DNA Separation Factor of at least 0.5.

In addition to the beads themselves being substantially metal-free, we have also found that, to achieve optimum peak separation, the separation

column and all process solutions held within the column or flowing through the column should be substantially free of multivalent cation contaminants. This can be achieved by supplying and feeding solutions entering the separation column with components which have process solution-contacting surfaces made of material which does not release multivalent cations into the process solutions held within or flowing through the column, in order to protect the column from multivalent cation contamination. The process solution-contacting surfaces of the system components are preferably material selected from the group consisting of titanium, coated stainless steel, and organic polymer.

For additional protection, multivalent cations in eluent solutions and sample solutions entering the column can be removed by contacting these solutions with multivalent cation capture resin before the solutions enter the column to protect the resin bed from multivalent cation contamination. The multivalent capture resin is preferably cation exchange resin and/or chelating resin.

The method of the present invention can be used to separate double stranded polynucleotides having up to about 1500 to 2000 base pairs. In many cases, the method is used to separate polynucleotides having up to 600 bases or base pairs, or which have up to 5 to 80 bases or base pairs. The mixture of polynucleotides can be a polymerase chain reaction product.

The method is performed at a temperature within the range of 20°C to 90°C to yield a back-pressure not greater than 5000 psi. The method also preferably employs an organic solvent that is water soluble. The solvent is preferably selected from the group consisting of alcohols, nitriles,

dimethylformamide, esters, and ethers. The method also preferably employs a counter ion agent selected from tialkylamine acetate, trialkylamine carbonate, and trialkylamine phosphate. The most preferred counter ion agent is triethylammonium acetate or triethylammonium hexafluoroisopropyl alcohol.

In another aspect the present invention provides a polymeric bead having an average bead diameter of 0.5-100 micron. Precautions are taken during the production of the beads so that they are substantially free of multivalent cation contaminants and the beads are treated, for example by an acid wash treatment, to remove any residual surface metal contaminants. In one embodiment, the beads are characterized by having a DNA Separation Factor of at least 0.05. In a preferred embodiment, the beads are characterized by having a DNA Separation Factor of at least 0.5. The bead preferably has an average diameter of about 1-10 microns, and most preferably has an average diameter of about 1-5 microns. The bead can be comprised of a copolymer of vinyl aromatic monomers. The vinyl aromatic monomers can be styrene, alkyl substituted styrene, alpha-methylstyrene or alkyl substituted alpha-methylstyrene. The bead can be a copolymer such as a copolymer of styrene, C_{1-6} alkyl vinylbenzene and divinylbenzene. The bead can contain functional groups such as polyvinyl alcohol, hydroxy, nitro, halogen (e.g. bromo), cyano, aldehyde, or other groups that do not bind the sample. The bead can be unsubstituted, or substituted with a moiety such as methyl, ethyl, hydrocarbon, or hydrocarbon polymer. In preferred embodiments, the bead is octadecyl modified poly(ethylvinylbenzene-

divinylbenzene) or poly(styrene-divinylbenzene). The bead can also contain crosslinking divinylmonomer such as divinyl benzene or butadiene.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of how the DNA Separation Factor is measured.

FIG. 2 is a MIPC separation of pUC18 DNA-*Hae*III digestion fragments on a column containing alkylated poly(styrene-divinylbenzene) beads. Peaks are labeled with the number of base pairs of the eluted fragment.

FIG. 3 is a MIPC separation of pUC18 DNA-*Hae*III digestion fragments on a column containing nonporous 2.1 micron beads of underivatized poly(styrene-divinylbenzene).

FIG. 4 is a Van't Hoff plot of ln k *vs.* $1/T\ [^{\circ}K^{-1}]$ with alkylated poly(styrene-divinylbenzene) beads showing positive enthalpy using acetonitrile as the solvent.

FIG. 5 is a Van't Hoff plot of ln k *vs.* $1/T\ [^{\circ}K^{-1}]$ with underivatized poly(styrene-divinylbenzene) beads showing positive enthalpy using acetonitrile as the solvent.

FIG. 6 is a Van't Hoff plot of ln k *vs.* $1/T\ [^{\circ}K^{-1}]$ with alkylated poly(styrene-divinylbenzene) beads showing negative enthalpy using methanol as the solvent.

FIG. 7 is a separation using alkylated beads and acetonitrile as solvent.

FIG. 8 is a separation using alkylated beads and 50.0% methanol as the solvent.

FIG. 9 is a separation using alkylated beads and 25.0% ethanol as the solvent.

FIG. 10 is a separation using alkylated beads and 25.0% vodka (100 proof) as the solvent.

FIG. 11 is a separation using alkylated beads and 25.0% 1-propanol as the solvent.

FIG. 12 is a separation using alkylated beads and 25.0% 1-propanol as the solvent.

FIG. 13 is a separation using alkylated beads and 10.0% 2-propanol as the solvent.

FIG. 14 is a separation using alkylated beads and 10.0% 2-propanol as the solvent.

FIG. 15 is a separation using alkylated beads and 25.0% THF as the solvent.

FIG. 16 is a combination isocratic/gradient separation on a non-alkylated poly(styrene-divinylbenzene) beads.

DETAILED DESCRIPTION OF THE INVENTION

In its most general form, the subject matter of the present invention is the separation of polynucleotides utilizing columns filled with nonporous polymeric beads having an average diameter of about 0.5 -100 microns; preferably, 1 - 10 microns; more preferably, 1 - 5 microns. Beads having an average diameter of 1.0 - 3.0 microns are most preferred.

In U.S. Patent No. 5,585,236, Bonn et al. had characterized the nucleic acid separation process as reverse phase ion pairing chromatography (RPIPC). However, since RPIPC does not incorporate certain essential

characteristics described in the present invention, another term, Matched Ion Polynucleotide Chromatography (MIPC), has been selected. MIPC as used herein, is defined as a process for separating single and double stranded polynucleotides using non-polar beads, wherein the process uses a counter ion agent, and an organic solvent to desorb the nucleic acid from the beads, and wherein the beads are characterized as having a DNA Separation Factor of at least 0.05. In a preferred embodiment, the beads have a DNA Separation Factor of at least 0.5. In an optimal embodiment, the beads have a DNA Separation Factor of at least 0.95.

The performance of the beads of the present invention is demonstrated by high efficiency separation by MIPC of double stranded and single stranded DNA. We have found that the best criterion for measuring performance of the beads is a DNA Separation Factor. This is measured as the resolution of 257- and 267-base pair double stranded DNA fragments of a pUC18 DNA-*Hae*III restriction digest wherein the distance from the valley between the peaks to the top of one of the peaks, over the distance from the baseline to the valley. Referring to the schematic representation of FIG. 1, the DNA Separation Factor is determined by measuring the distance "**a**" from the baseline to the valley "**e**" between the peaks "**b**" and "**c**" and the distance "**d**" from the valley "**e**" to the top of one of the peaks "**b**" or "**c**". If the peak heights are unequal, the highest peak is used to obtain "**d.**" The DNA Separation Factor is the ratio of **d/(a+d)**. The peaks of 257- and 267-base pairs in this schematic representation are similar in height. Operational beads of the present invention have a DNA Separation Factor of at least 0.05. Preferred beads have a DNA Separation Factor of at least 0.5.

Without wishing to be bound by theory, we believe that the beads which conform to the DNA Separation Factor as specified herein have a pore size which essentially excludes the polynucleotides being separated from entering the bead. As used herein, the term "nonporous" is defined to denote a bead which has surface pores having a diameter that is less than the size and shape of the smallest DNA fragment in the separation in the solvent medium used therein. Included in this definition are polymer beads having these specified maximum size restrictions in their natural state or which have been treated to reduce their pore size to meet the maximum effective pore size required. Preferably, all beads which provide a DNA Separation Factor of at least 0.05 are intended to be included within the definition of "nonporous" beads.

The surface conformations of nonporous beads of the present invention can include depressions and shallow pit-like structures which do not interfere with the separation process. A pretreatment of a porous bead to render it nonporous can be effected with any material which will fill the pores in the bead structure and which does not significantly interfere with the MIPC process.

Pores are open structures through which eluent and other materials can enter the bead structure. Pores are often interconnected so that fluid entering one pore can exit from another pore. We believe that pores having dimensions that allow movement of the polynucleotide into the interconnected pore structure and into the bead impair the resolution of separations or result separations that have very long retention times. In MIPC, however, the beads are "nonporous" and the polynucleotides do not enter the bead structure.

The term polynucleotide is defined as a linear polymer containing an indefinite number of nucleotides, linked from one ribose (or deoxyribose) to another via phosphoric residues. The present invention can be used in the separation of RNA or of double- or single-stranded DNA. For purposes of simplifying the description of the invention, and not by way of limitation, the separation of double-stranded DNA will be described in the examples herein, it being understood that all polynucleotides are intended to be included within the scope of this invention.

Chromatographic efficiency of the column beads is predominantly influenced by the properties of surface and near-surface areas. For this reason, the following descriptions are related specifically to the close-to-the-surface region of the polymeric beads. The main body and/or the center of such beads can exhibit entirely different chemistries and sets of physical properties from those observed at or near the surface of the polymeric beads of the present invention.

The nonporous polymeric beads of the present invention are prepared by a two-step process in which small seed beads are initially produced by emulsion polymerization of suitable polymerizable monomers. The emulsion polymerization procedure of the invention is a modification of the procedure of Goodwin et al. (J. W. Goodwin, J. Hearn, C. C. Ho and R. H. Ottweill, Colloid & Polymer Sci., (1974), 252:464-471). Monomers which can be used in the emulsion polymerization process to produce the seed beads include styrene, alkyl substituted styrenes, alpha-methyl styrene, and alkyl substituted alpha-methyl styrene. The seed beads are then enlarged and, optionally, modified

by substitution with various groups to produce the nonporous polymeric beads of the present invention.

The seed beads produced by emulsion polymerization can be enlarged by any known process for increasing the size of the polymer beads. For example, polymer beads can be enlarged by the activated swelling process disclosed in U.S. Patent No. 4,563,510. The enlarged or swollen polymer beads are further swollen with a crosslinking polymerizable monomer and a polymerization initiator. Polymerization increases the crosslinking density of the enlarged polymeric bead and reduces the surface porosity of the bead. Suitable crosslinking monomers contain at least two carbon-carbon double bonds capable of polymerization in the presence of an initiator. Preferred crosslinking monomers are divinyl monomers, preferably alkyl and aryl (phenyl, naphthyl, etc.) divinyl monomers and include divinyl benzene, butadiene, etc. Activated swelling of the polymeric seed beads is useful to produce polymer beads having an average diameter ranging from 1 up to about 100 microns.

Alternatively, the polymer seed beads can be enlarged simply by heating the seed latex resulting from emulsion polymerization. This alternative eliminates the need for activated swelling of the seed beads with an activating solvent. Instead, the seed latex is mixed with the crosslinking monomer and polymerization initiator described above, together with or without a water-miscible solvent for the crosslinking monomer. Suitable solvents include acetone, tetrahydrofuran (THF), methanol, and dioxane. The resulting mixture is heated for about 1 - 12 hours, preferably about 4 - 8 hours, at a temperature below the initiation temperature of the polymerization

initiator, generally, about 10°C - 80°C, preferably 30°C - 60°C. Optionally, the temperature of the mixture can be increased by 10 - 20% and the mixture heated for an additional 1 to 4 hours. The ratio of monomer to polymerization initiator is at least 100:1, preferably about 100:1 to about 500:1, more preferably about 200:1 in order to ensure a degree of polymerization of at least 200. Beads having this degree of polymerization are sufficiently pressure-stable to be used in high pressure liquid chromatography (HPLC) applications. This thermal swelling process allows one to increase the size of the bead by about 110 - 160% to obtain polymer beads having an average diameter up to about 5 microns, preferably about 2 - 3 microns. The thermal swelling procedure can, therefore, be used to produce smaller particle sizes previously accessible only by the activated swelling procedure.

Following thermal enlargement, excess crosslinking monomer is removed and the particles are polymerized by exposure to ultraviolet light or heat. Polymerization can be conducted, for example, by heating of the enlarged particles to the activation temperature of the polymerization initiator and continuing polymerization until the desired degree of polymerization has been achieved. Continued heating and polymerization allows one to obtain beads having a degree of polymerization greater than 500.

In the present invention, the packing material disclosed by Bonn et al. or U.S. Patent No. 4,563,510 can be modified through substitution of the polymeric beads with methyl or ethyl groups or with a hydrocarbon polymer group or can be used in its unmodified state. The polymer beads can be alkylated with 1 or 2 carbon atoms by contacting the beads with an alkylating agent, such as methyl iodide or ethyl iodide. Alkylation is achieved by mixing

the polymer beads with the alkyl halide in the presence of a Friedel-Crafts catalyst to effect electrophilic aromatic substitution on the aromatic rings at the surface of the polymer blend. Suitable Friedel-Crafts catalysts are well-known in the art and include Lewis acids such as aluminum chloride, boron trifluoride, tin tetrachloride, etc. The beads can be hydrocarbon substituted by substituting the corresponding hydrocarbon halide for methyl iodide in the above procedure, for example.

The term "hydrocarbon" as used herein is defined to include alkyl and alkyl substituted aryl groups, having from 23 to 1,000,000 carbons, the alkyl groups including straight chained, branch chained, cyclic, saturated, unsaturated nonionic functional groups of various types including aldehyde, ketone, ester, ether, alkyl groups, and the like, and the aryl groups including as monocyclic, bicyclic, and tricyclic aromatic hydrocarbon groups including phenyl, naphthyl, and the like. Methods for hydrocarbon substitution are conventional and well-known in the art and are not an aspect of this invention. The substitution can also contain hydroxy, cyano, nitro groups, or the like which are considered to be non-polar, reverse phase functional groups. The term "hydrocarbon polymer" as used herein is defined to be a polymer having a hydrocarbon composition and from 23 to 1,000,000 carbons.

The chromatographic material reported in the Bonn patent was limited to nonporous beads substituted with alkyl groups having at least 3 carbons because Bonn et al. were unsuccessful in obtaining separations using polymer beads lacking this substitution. Additionally, the polymer beads were limited to a small group of vinyl aromatic monomers, and Bonn et al. were unable to effect double stranded DNA separations with other materials.

In the present invention, it has now been surprisingly discovered that successful separation of double stranded DNA can be achieved using underivatized nonporous beads as well as using beads derivatized with methyl, ethyl, hydrocarbon, or hydrocarbon polymer substitution.

The base polymer of the invention can also be other polymers, non-limiting examples of which include mono- and di-vinyl substituted aromatics such as styrene, substituted styrenes, alpha-substituted styrenes and divinylbenzene; acrylates and methacrylates; polyolefins such as polypropylene and polyethylene; polyesters; polyurethanes; polyamides; polycarbonates; and substituted polymers including fluorosubstituted ethylenes commonly known under the trademark TEFLON. The base polymer can also be mixtures of polymers, non-limiting examples of which include poly(styrene-divinylbenzene) and poly(ethylvinylbenzene-divinylbenzene). Methods for making beads from these polymers are conventional and well known in the art (for example, see U.S. Patent 4,906,378). The physical properties of the surface and near-surface areas of the beads are the predominant influence on chromatographic efficiency. The polymer, whether derivatized or not, must provide a nonporous, non-reactive, and non-polar surface for the Matched Ion Polynucleotide Chromatographic separation.

The beads of the invention are also characterized by having low amounts of metal contaminants or other contaminants that can bind DNA. The preferred beads of the present invention are characterized by having been subjected to precautions during production, including a decontamination treatment, such as an acid wash treatment, designed to substantially

eliminate any multivalent cation contaminants (e.g. Fe(III), Cr(III), or colloidal metal contaminants). Only very pure, non-metal containing materials should be used in the production of the beads in order that the resulting beads will have minimum metal content.

In addition to the beads themselves being substantially metal-free, we have also found that, to achieve optimum peak separation during MIPC, the separation column and all process solutions held within the column or flowing through the column should be substantially free of multivalent cation contaminants. As described in commonly owned, copending U.S. application Ser. No. 08/748,376 (filed November 13, 1996), this can be achieved by supplying and feeding solutions entering the separation column with components which have process solution-contacting surfaces made of material which does not release multivalent cations into the process solutions held within or flowing through the column, in order to protect the column from multivalent cation contamination. The process solution-contacting surfaces of the system components are preferably material selected from the group consisting of titanium, coated stainless steel, passivated stainless steel, and organic polymer.

For additional protection, multivalent cations in eluent solutions and sample solutions entering the column can be removed by contacting these solutions with multivalent cation capture resin before the solutions enter the column to protect the resin bed from multivalent cation contamination. The multivalent capture resin is preferably cation exchange resin and/or chelating resin.

To achieve high resolution chromatographic separations of polynucleotides, it is generally necessary to tightly pack the chromatographic column with the solid phase polymer beads. Any known method of packing the column with a column packing material can be used in the present invention to obtain adequate high resolution separations. Typically, a slurry of the polymer beads is prepared using a solvent having a density equal to or less than the density of the polymer beads. The column is then filled with the polymer bead slurry and vibrated or agitated to improve the packing density of the polymer beads in the column. Mechanical vibration or sonification are typically used to improve packing density.

For example, to pack a 4.6 x 50 mm i.d. column, 2.0 grams of beads can be suspended in 10 mL of methanol with the aid of sonification. The suspension is then packed into the column using 50 mL of methanol at 8,000 psi of pressure. This improves the density of the packed bed.

The separation method of the invention is generally applicable to the chromatographic separation of single stranded and double stranded polynucleotides of DNA and RNA. Samples containing mixtures of polynucleotides can result from total synthesis of polynucleotides, cleavage of DNA or RNA with restriction endonucleases or with other enzymes or chemicals, as well as nucleic acid samples which have been multiplied and amplified using polymerase chain reaction techniques.

The method of the present invention can be used to separate double stranded polynucleotides having up to about 1500 to 2000 base pairs. In many cases, the method is used to separate polynucleotides having up to 600 bases or base pairs, or which have up to 5 to 80 bases or base pairs.

In a preferred embodiment, the separation is by Matched Ion Polynucleotide Chromatography (MIPC). The nonporous beads of the invention are used as a reverse phase material that will function with counter ion agents and a solvent gradient to produce the DNA separations. In MIPC, the polynucleotides are paired with a counter ion and then subjected to reverse phase chromatography using the nonporous beads of the present invention. Counter ion agents that are volatile, such as trialkylamine acetate, trialkylamine carbonate, trialkylamine phosphate ($TEAAHPO_4$), etc., are preferred for use in the method of the invention, with triethylammonium acetate (TEAA) and triethylammonium hexafluoroisopropyl alcohol being most preferred.

To achieve optimum peak resolution during the separation of DNA by MIPC using the beads of the invention, the method is preferably performed at a temperature within the range of 20°C to 90°C; more preferably, 30°C to 80°C; most preferably, 50°C to 75°C. 14. The temperature is selected to yield a back pressure not exceeding 5000 psi. In general, separation of single-stranded fragments should be performed at higher temperatures.

We have found that the temperature at which the separation is performed affects the choice of solvents used in the separation. One reason is that the solvents affect the temperature at which a double stranded DNA will melt to form two single strands or a partially melted complex of single and double stranded DNA. Some solvents can stabilize the melted structure better than other solvents. The other reason a solvent is important is because it affects the transport of the DNA between the mobile phase and the stationary phase. Acetonitrile and 1-propanol are preferred solvents in these

cases. Finally, the toxicity (and cost) of the solvent can be important. In this case, methanol is preferred over acetonitrile and 1-propanol is preferred over methanol.

When the separation is performed at a temperature within the above range, an organic solvent that is water soluble is preferably used, for example, alcohols, nitriles, dimethylformamide (DMF), tetrahydrofuran (THF), esters, and ethers. Water soluble solvents are defined as those which exist as a single phase with aqueous systems under all conditions of operation of the present invention. Solvents which are particularly preferred for use in the method of this invention include methanol, ethanol, 2-propanol, 1-propanol, tetrahydrofuran (THF), and acetonitrile, with acetonitrile being most preferred overall.

We have determined that the chromatographic separations of double stranded DNA fragments exhibit unique Sorption Enthalpies (ΔH_{sorp}). Two compounds (in this case, DNA fragments of different size) can only be separated if they have different partition coefficients (K). The Nernst partition coefficient is defined as the concentration of an analyte (A) in the stationary phase divided by its concentration in the mobile phase:

$$K = \frac{[A]_s}{[A]_m}$$

The partition coefficient (K) and the retention factor (k) are related through the following equations:

$$K = \frac{n(A)_s V_m}{n(A)_m V_s} \quad \text{and} \quad k = \frac{n(A)_s}{n(A)_m}$$

the quotient V_m/V_s is also called phase volume ratio (Φ). Therefore:

$$k = K\Phi$$

To calculate the sorption enthalpies, the following fundamental thermodynamic equations are necessary:

$$\ln K = -\frac{\Delta G_{sorp}}{RT}, \quad \ln k = -\frac{\Delta G_{sorp}}{RT} + \ln \Phi \quad \text{and} \quad \Delta G_{sorp} = \Delta H_{sorp} - T\Delta S_{sorp}$$

By transforming the last two equations, we obtain the Van't Hoff equation:

$$\ln k = -\frac{\Delta H_{sorp}}{RT} + \frac{\Delta S_{sorp}}{R} + \ln \Phi$$

From a plot ln k versus $1/T$, the sorption enthalpy ΔH_{sorp} can be obtained from the slope of the graph (if a straight line is obtained). ΔS_{sorp} can be calculated if the phase volume ratio (Φ) is known.

The Sorption Enthalpy ΔH_{sorp} is positive ($\Delta H_{sorp} > 0$) showing the separation is endothermic using acetonitrile as the solvent (Figs. 3 and 4), and using methanol as the solvent, the Sorption Enthalpy ΔH_{sorp} is negative ($\Delta H_{sorp} < 0$), showing the separation is exothermic (FIG. 5).

The thermodynamic data (as shown in the Examples hereinbelow) reflect the relative affinity of the DNA-counter ion agent complex for the beads of the invention and the elution solvent. An endothermic plot indicates a preference of the DNA complex for the bead. An exothermic plot indicates a preference of the DNA complex for the solvent over the bead. The plots shown herein are for alkylated and non-alkylated surfaces as described in the Examples. Most liquid chromatographic separations show exothermic plots.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

All references cited herein are hereby incorporated by reference in their entirety.

Procedures described in the past tense in the examples below have been carried out in the laboratory. Procedures described in the present tense have not yet been carried out in the laboratory, and are constructively reduced to practice with the filing of this application.

EXAMPLE 1

Preparation of nonporous poly(styrene-divinylbenzene) particles

Sodium chloride (0.236 g) was added to 354 mL of deionized water in a reactor having a volume of 1.0 liter. The reactor was equipped with a mechanical stirrer, reflux condenser, and a gas introduction tube. The dissolution of the sodium chloride was carried out under inert atmosphere (argon), assisted by stirring (350 rpm), and at an elevated temperature (87°C). Freshly distilled styrene (33.7 g) and 0.2184 g of potassium peroxodisulfate ($K_2S_2O_8$) dissolved in 50 mL of deionized water were then added. Immediately after these additions, the gas introduction tube was pulled out of the solution and positioned above the liquid surface. The reaction mixture was subsequently stirred for 6.5 hours at 87°C. After this, the contents of the reactor were cooled down to ambient temperature and diluted to a volume yielding a concentration of 54.6 g of polymerized styrene in 1000 mL volume of suspension resulting from the first step. The amount of polymerized styrene in 1000 mL was calculated to include the quantity of the polymer still sticking to the mechanical stirrer (approximately 5 - 10 g). The diameter of the spherical beads in the suspension was determined by light microscopy to be about 1.0 micron.

Beads resulting from the first step are still generally too small and too soft (low pressure stability) for use as chromatographic packings. The softness of these beads is caused by an insufficient degree of crosslinking. In a second step, the beads are enlarged and the degree of crosslinking is increased.

The protocol for the second step is based on the activated swelling method described by Ugelstad et al. (*Adv. Colloid Interface Sci.*, 13:101-140 (1980)). In order to initiate activated swelling, or the second synthetic step, the aqueous suspension of polystyrene seeds (200 ml) from the first step was mixed first with 60 mL of acetone and then with 60 mL of a 1-chlorododecane emulsion. To prepare the emulsion, 0.206 g of sodium dodecylsulfate, 49.5 mL of deionized water, and 10.5 mL of 1-chlorododecane were brought together and the resulting mixture was kept at 0°C for 4 hours and mixed by sonication during the entire time period until a fine emulsion of < 0.3 microns was obtained. The mixture of polystyrene seeds, acetone, and 1-chlorododecane emulsion was stirred for about 12 hours at room temperature, during which time the swelling of the beads occurred. Subsequently, the acetone was removed by a 30 minute distillation at 80°C.

Following the removal of acetone, the swollen beads were further grown by the addition of 310 g of a ethyldivinylbenzene and divinylbenzene (DVB) (1:1.71) mixture also containing 2.5 g of dibenzoylperoxide as an initiator. The growing occurred with stirring and with occasional particle size measurements by means of light microscopy.

After completion of the swelling and growing stages, the reaction mixture was transferred into a separation funnel. In an unstirred solution, the

excess amount of the monomer separated from the layer containing the suspension of the polymeric beads and could thus be easily removed. The remaining suspension of beads was returned to the reactor and subjected to a stepwise increase in temperature (63°C for about 7 hours, 73°C for about 2 hours, and 83°C for about 12 hours), leading to further increases in the degree of polymerization (> 500). The pore size of beads prepared in this manner was below the detection limit of mercury porosimetry (< 30Å).

After drying, the dried beads (10 g) from step two were washed four times with 100 mL of n-heptane, and then two times with each of the following: 100 mL of diethylether, 100 mL of dioxane, and 100 mL of methanol. Finally, the beads were dried.

EXAMPLE 2

Acid Wash Treatment

The beads prepared in Example 1 were washed three times with tetrahydrofuran and two times with methanol. Finally the beads were stirred in a mixture containing 100 mL tetrahydrofuran and 100 mL concentrated hydrochloric acid for 12 hours. After this acid treatment, the polymer beads were washed with a tetrahydrofuran/water mixture until neutral (pH = 7). The beads were then dried at 40°C for 12 hours.

EXAMPLE 3

Standard Procedure for Testing the Performance of Separation Media

Separation particles are packed in an HPLC column and tested for their ability to separate a standard DNA mixture. The standard mixture is a pUC18 DNA-*Hae*III digest (Sigma-Aldrich, D6293) which contains 11 fragments having 11, 18, 80, 102, 174, 257, 267, 298, 434, 458, and 587

base pairs, respectively. The standard is diluted with water and five µL, containing a total mass of DNA of 0.25 µg, is injected.

Depending on the packing volume and packing polarity, the procedure requires selection of the driving solvent concentration, pH, and temperature. The separation conditions are adjusted so that the retention time of the 257, 267 peaks is about 6 to 10 minutes. Any one of the following solvents can be used: methanol, ethanol, 2-propanol, 1-propanol, tetrahydrofuran (THF), or acetonitrile. A counter ion agent is selected from trialkylamine acetate, trialkylamine carbonate, trialkylamine phosphate, or any other type of cation that can form a matched ion with the polynucleotide anion.

As an example of this procedure, FIG. 2 shows the high resolution of the standard DNA mixture using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads. The separation was conducted under the following conditions: Eluent A: 0.1 M TEAA, pH 7.0; Eluent B: 0.1 M TEAA, 25% acetonitrile; Gradient:

Time (min)	%A	%B
0.0	65	35
3.0	45	55
10.0	35	65
13.0	35	65
14.0	0	100
15.5	0	100
16.5	65	35

The flow rate was 0.75 mL/min, detection UV at 260 nm, column temp. 50°C. The pH was 7.0.

As another example of this procedure using the same separation conditions as in FIG. 2, FIG. 3 is a high resolution separation of the standard DNA mixture on a column containing nonporous 2.1 micron beads of underivatized poly(styrene-divinylbenzene).

EXAMPLE 4

Sorption Enthalpy Measurements

Four fragments (174 bp, 257 bp, 267 bp, and 298 bp, found in 5 µL pUC18 DNA-*Hae*III digest, 0.04 µg DNA/µL) were separated under isocratic conditions at different temperatures using octadecyl modified, nonporous poly(styrene-divinylbenzene) polymer beads. The separation was carried out using a Transgenomic WAVE™ DNA Fragment Analysis System equipped with a DNASep™ column (Transgenomic, Inc., San Jose, CA) under the following conditions: Eluent: 0.1 M triethylammonium acetate, 14.25% (v/v) acetonitrile at 0.75 mL/min, detection at 250 nm UV, temperatures at 35, 40, 45, 50, 55, and 60°C, respectively. A plot of ln *k* versus *1/T* shows that the retention factor *k* is increasing with increasing temperature (FIG. 4). This indicates that the retention mechanism is based on an endothermic process ($\Delta H_{sorp} > 0$).

The same experiments on non-alkylated poly(styrene-divinylbenzene) beads gave a negative slope for a plot of ln *k* versus *1/T*, although the plot is slightly curved (FIG. 5).

The same experiments performed on octadecyl modified, nonporous poly(styrene-divinylbenzene) beads but with methanol replacing the acetonirile as solvent gave a plot ln *k* versus *1/T* showing the retention factor

k is decreasing with increasing temperature (FIG. 6). This indicates the retention mechanism is based on an exothermic process ($\Delta H_{sorp} < 0$).

EXAMPLE 5

Separations with alkylated poly(styrene-divinylbenzene) beads

Eluents are chosen to match the desorption ability of the elution solvent to the attraction properties of the bead to the DNA-counter ion complex. As the polarity of the bead decreases, a stronger (more organic) or higher concentration of solvent will be required. Weaker organic solvents such as methanol are generally required at higher concentrations than stronger organic solvents such as acetonitrile.

FIG. 7 shows the high resolution separation of DNA restriction fragments using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads. The experiment was conducted under the following conditions: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.2; gradient: 33-55% acetonitrile in 3 min, 55-66% acetonitrile in 7 min, 65% acetonitrile for 2.5 min; 65-100% acetonitrile in 1 min; and 100-35% acetonitrile in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, column temp. 51°C. The sample was 5 µL (= 0.2 µg pUC18 DNA-*Hae*III digest).

Repeating the procedure of FIG. 7 replacing the acetonitrile with 50.0% methanol in 0.1 M (TEAA) gave the separation shown in FIG. 8.

Repeating the procedure of FIG. 7 replacing the acetonitrile with 25.0% ethanol in 0.1 M (TEAA) gave the separation shown in FIG. 9.

Repeating the procedure of FIG. 7 replacing the acetonitrile with 25% vodka (Stolichnaya, 100 proof) in 0.1 M (TEAA) gave the separation shown in FIG. 10.

The separation shown in FIG. 11 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M tetraethylacetic acid (TEAA), pH 7.3; gradient: 12-18% 0.1 M TEAA and 25.0% 1-propanol (Eluent B) in 3 min, 18-22% B in 7 min, 22% B for 2.5 min; 22-100% B in 1 min; and 100-12% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µL (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 12 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 15-18% 0.1 M TEAA and 25.0% 1-propanol (Eluent B) in 2 min, 18-21% B in 8 min, 21% B for 2.5 min; 21-100% B in 1 min; and 100-15% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µL (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 13 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 35-55% 0.1 M TEAA and 10.0% 2-propanol (Eluent B) in 3 min, 55-65 % B in 10 min, 65% B for 2.5 min; 65-100% B in 1 min; and 100-35% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µL (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 14 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEA_2HPO_4, pH 7.3; gradient: 35-55% 0.1 M TEA_2HPO_4 and 10.0% 2-propanol (Eluent B) in 3 min, 55-65% B in 7 min, 65% B for 2.5 min; 65-100% B in 1 min; and 100-65% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µL (= 0.2 µg pUC18 DNA-*Hae*III digest).

The separation shown in FIG. 15 was obtained using octadecyl modified, nonporous poly(ethylvinylbenzene-divinylbenzene) beads as follows: Column: 50 x 4.6 mm i.d.; mobile phase 0.1 M TEAA, pH 7.3; gradient: 6-9% 0.1 M TEAA and 25.0% THF (Eluent B) in 3 min, 9-11 % B in 7 min, 11% B for 2.5 min; 11-100% B in 1 min; and 100-6% B in 1.5 min. The flow rate was 0.75 mL/min, detection UV at 260 nm, and column temp. 51°C. The sample was 5 µL (= 0.2 µg pUC18 DNA-*Hae*III digest).

EXAMPLE 6

Isocratic/gradient separation of ds DNA

The following is an isocratic/gradient separation of ds DNA using nonporous poly(styrene-divinylbenzene) beads. Isocratic separations have not been performed in DNA separations because of the large differences in the selectivity of DNA/alkylammonium ion pair for beads. However, by using a combination of gradient and isocratic elution conditions, the resolving power of a system can be enhanced for a particular size range of DNA. For example, the range of 250-300 base pairs can be targeted by using an eluent of 0.1 M TEAA, and 14.25% acetonitrile at 0.75 mL/min at 40°C on 50 x 4.6

mm cross-linked poly(styrene-divinylbenzene) column, 2.1 micron. 5 µL of pUC18 DNA-*Hae*III digest (0.2 µg) was injected under isocratic conditions and 257, 267 and 298 base pairs DNA eluted completely resolved as shown in FIG. 16. Then the column was cleaned from larger fragments with 0.1M TEAA/25% acetonitrile at 9 minutes. In other examples, there might be an initial isocratic step (to condition the column), then a gradient step (to remove or target the first group of DNA at a particular size), then an isocratic step (to separate the target material of a different size range) and finally a gradient step to clean the column.

EXAMPLE 7

Bromination of Remaining Double Bonds on the Surface of Poly(Styrene-Divinylbenzene) Polymer Beads

50.0 g of a poly(styrene-divinylbenzene) polymer beads were suspended in 500 g of tetrachloromethane. The suspension was transferred into a 1000 mL glass reactor (with attached reflux condenser, separation funnel and overhead stirrer). The mixture was kept at 20°C. Bromine (100 mL) was added over a period of 20 minutes. After addition was completed, stirring continued for 60 minutes. The temperature was raised to 50°C to complete the reaction (2 hours).

The polymer beads were separated from the tetrachloromethane and excess bromine by means of centrifugation and cleaned with tetrahydrofuran (once with 100 mL) and methanol (twice with 100 mL). The polymer beads were dried at 40°C.

The polymer beads are packed into a 50 x 4.6 mm i.d column and the DNA Separation Factor is greater than 0.05 as tested by the procedure of Example 3.

EXAMPLE 8

Nitration of a Poly(Styrene-Divinylbenzene) Polymer Beads

In a 1000 mL glass reactor 150 mL of concentrated nitric acid (65%) were combined with 100 mL concentrated sulfuric acid. The acid mixture was cooled to 0-4°C. When the temperature had dropped to <4°C, 50 g of poly(styrene-divinylbenzene) polymer beads were added slowly under continuous stirring. After addition was completed, 50 mL of nitric acid (65%) was added. The suspension was stirred for three hours, maintaining a temperature of 5-10°C.

On the next day the reaction was quenched by adding ice to the suspension. The polymer beads were separated from the acid by means of centrifugation. The polymer beads were washed to neutrality with water, followed by washing steps with tetrahydrofurane (four times with 100 mL) and methanol (four times with 100 mL). The polymer beads were dried at 40°C.

The polymer beads are packed into a 50 x 4.6 mm i.d column and the DNA Separation Factor is greater than 0.05 as tested by the procedure of Example 3.

While the foregoing has presented specific embodiments of the present invention, it is to be understood that these embodiments have been presented by way of example only. It is expected that others will perceive and practice variations which, though differing from the foregoing, do not

depart from the spirit and scope of the invention as described and claimed herein.

The invention claimed is:

1. A method for separating a mixture of polynucleotides, comprising flowing a mixture of polynucleotides having up to 1500 base pairs through a separation column containing polymer beads having an average diameter of 0.5 to 100 microns, said beads having a surface composition essentially completely substituted with a moiety selected from the group consisting of unsubstituted, methyl, ethyl, hydrocarbon, and hydrocarbon polymer, and wherein said beads are characterized by having a DNA Separation Factor of at least 0.05; and separating said mixture of polynucleotides.

2. The method of Claim 1 wherein said beads are characterized by having a DNA Separation Factor of at least 0.5.

3. The method of Claim 1 wherein said beads are substituted with a moiety selected from the group consisting of methyl, ethyl, hydrocarbon, and hydrocarbon polymer, wherein said hydrocarbon polymer optionally has from 23 to 1,000,000 carbons.

4. The method of Claim 3 wherein said moiety is substituted with a functional group selected from the group consisting of polyvinyl alcohol, hydroxy, nitro, bromo, cyano, and aldehyde.

5. The method of Claim 1, wherein said separation is by Matched Ion Polynucleotide Chromatography.

6. The method of claim 1 wherein said beads are subjected to an acid wash treatment.

7. The method of claim 1 wherein said beads are substantially free of multivalent cation contaminants.

8. The method of Claim 1, wherein said beads have an average diameter of about 1 - 5 microns.

9. The method of Claim 1, wherein said beads are comprised of a polymer selected from mono- and di-vinyl substituted aromatic compounds, acrylates, methacrylates, polyolefins, polyesters, polyurethanes, polyamides, polycarbonates, and flurosubstituted ethylenes.

10. The method of Claim 1, wherein said beads are substituted with hydrocarbon groups or substituted hydrocarbon groups.

11. The method of Claim 1, wherein said polynucleotides comprise RNA.

12. The method of Claim 1, wherein said polynucleotides comprise DNA.

13. The method of Claim 1, wherein said mixture of polynucleotides is a polymerase chain reaction product.

14. The method of Claim 1, wherein said method is performed at a temperature within the range of 20°C to 90°C.

15. The method of Claim 14, wherein said temperature is selected to yield a back pressure not exceeding 5000 psi.

16. The method of Claim 1, wherein said method employs an organic solvent, wherein said organic solvent is water soluble.

17. The method of Claim 16, wherein said solvent is selected from the group consisting of alcohols, nitriles, dimethylformamide, tetrahydrofuran, esters, and ethers.

18. The method of claim 1 wherein the mixture of polynucleotides includes a counter ion agent selected from trialkylamine acetate, trialkylamine carbonate and trialkylamine phosphate.

19. The method of claim 18 wherein the counter ion agent is selected from triethylammonium acetate and triethylammonium hexafluoroisopropyl alcohol.

20. A polymeric bead having an average bead diameter of 0.5-100 micron and said bead characterized by having a DNA Separation Factor of at least 0.05.

21. The bead of claim 20 wherein said bead has a DNA Separation Factor of at least 0.5.

22. The bead of claim 20 wherein said bead is subjected to an acid wash treatment.

23. The bead of claim 20 wherein said bead is substantially free of multivalent cations.

24. The bead of claim 20 wherein said bead has an average diameter of about 1-10 microns.

25. The bead of claim 20 wherein said bead has an average diameter of about 1-5 microns.

26. The bead of claim 20 wherein said bead is comprised of a copolymer of vinyl aromatic monomers.

27. The bead of claim 26 wherein said vinyl aromatic monomers are selected from the group consisting of styrene, alkyl substituted styrene, alpha-methylstyrene and alkyl substituted alpha-methylstyrene.

28. The bead of claim 26 wherein said bead is comprised of a copolymer of styrene, C_{1-6} alkyl vinylbenzene and divinylbenzene.

29. The polymer bead of claim 20, wherein said bead is substituted with a functional group selected from the group consisting of polyvinyl alcohol, hydroxy, nitro, bromo, cyano, and aldehyde.

30. The bead of claim 20 wherein said bead is substituted with a functional group selected from group consisting of methyl, ethyl, hydrocarbon, and hydrocarbon polymer.

31. The bead of claim 20 wherein said bead comprises poly(styrene-divinylbenzene).

32. The polymer bead of claim 20, wherein said bead comprises a polymer selected from mono- and di-vinyl substituted aromatic compounds, acrylates, methacrylates, polyolefins, polyesters, polyurethanes, polyamides, polycarbonates, and flurosubstituted ethylenes.

33. The polymer bead of claim 32, wherein said bead is substituted with a substituent selected from the group consisting of methyl, ethyl, hydrocarbon, and hydrocarbon polymer.

34. The polymer bead of claim 32, wherein said bead is substituted with a substituent selected from the group consisting of hydrocarbon and substituted hydrocarbon.

35. The bead of claim 20 and further including a crosslinking divinylmonomer selected from divinyl benzene and butadiene.

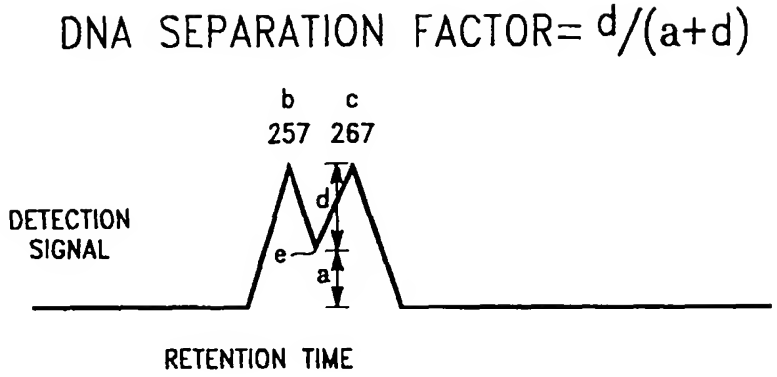

DNA SEPARATION FACTOR= d/(a+d)
b
257
c
267
d
e
a
DETECTION SIGNAL
RETENTION TIME

FIG.—1

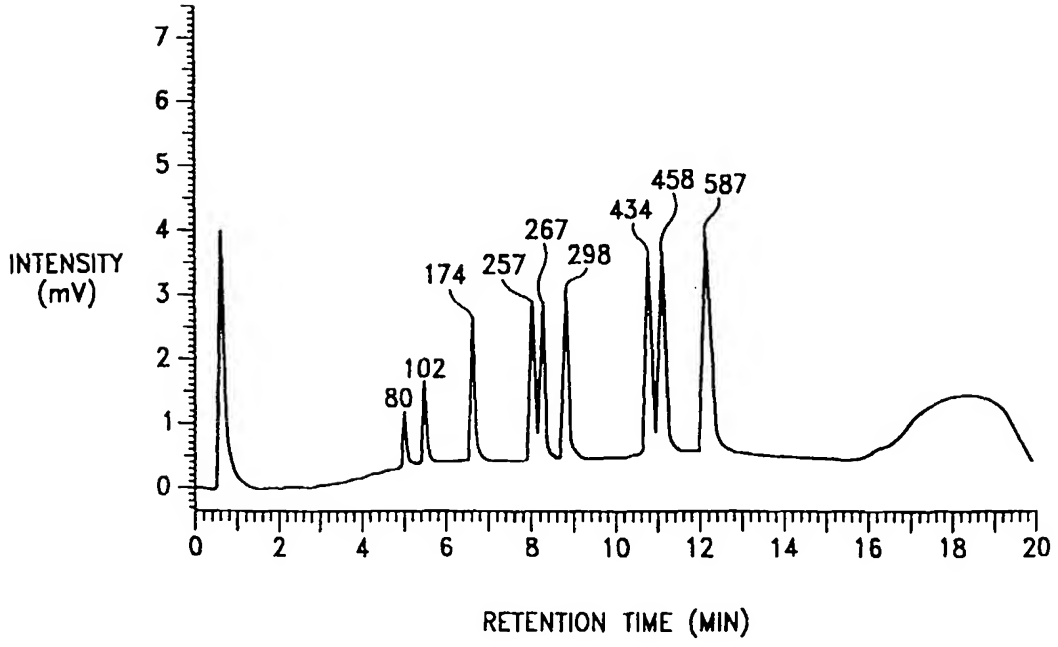

INTENSITY (mV)
0
1
2
3
4
5
6
7
80
102
174
257
267
298
434
458
587
0
2
4
6
8
10
12
14
16
18
20
RETENTION TIME (MIN)

FIG.—2

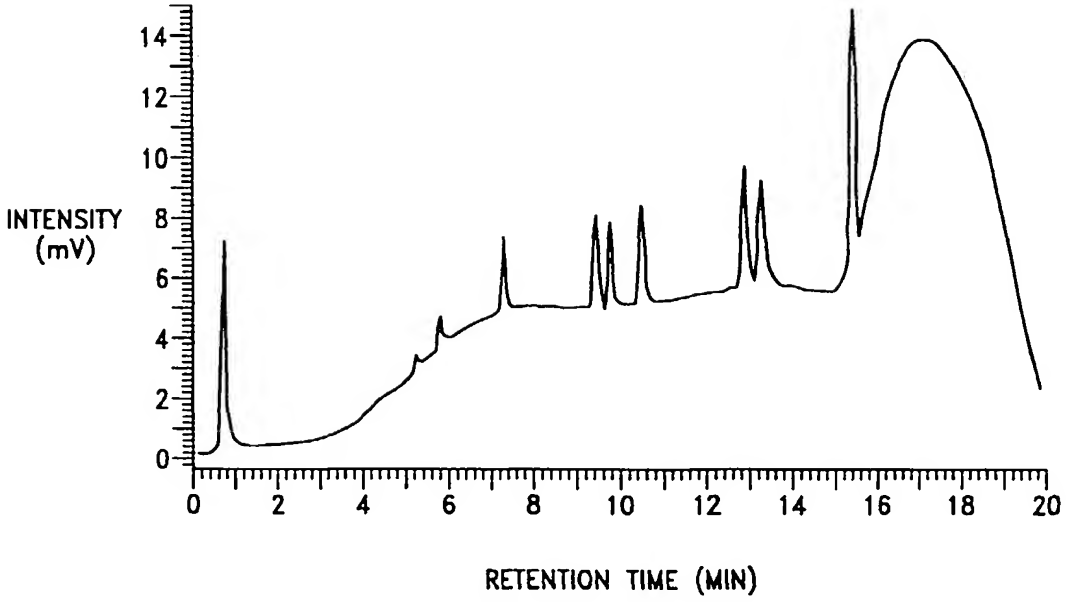

INTENSITY (mV)
0
2
4
6
8
10
12
14
0
2
4
6
8
10
12
14
16
18
20
RETENTION TIME (MIN)

FIG.—3

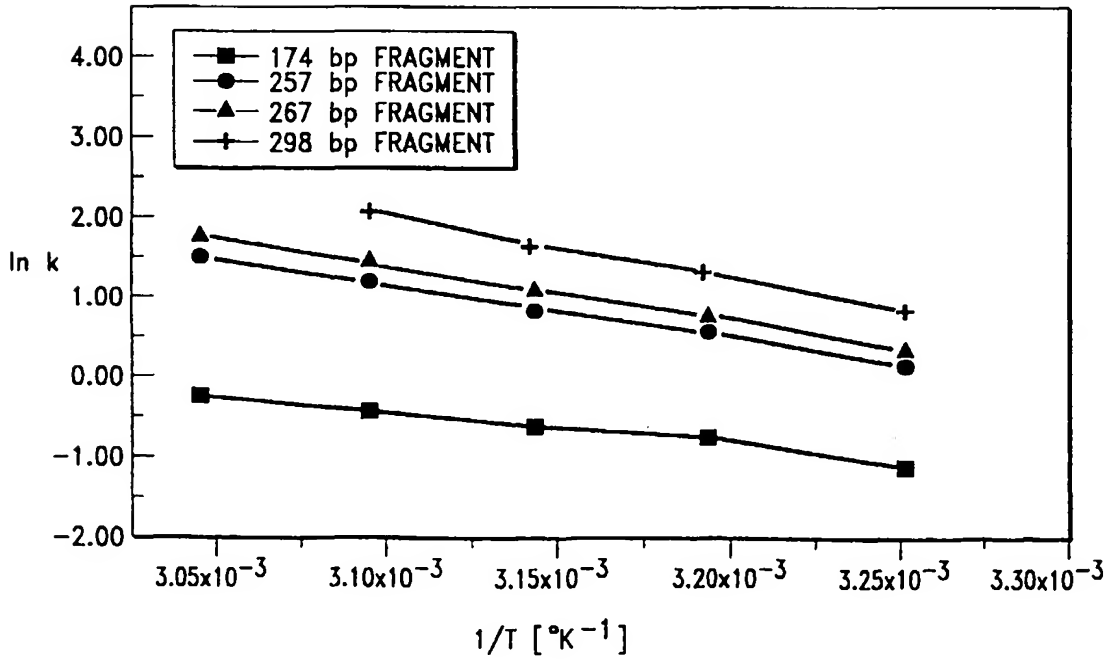

174 bp FRAGMENT
257 bp FRAGMENT
267 bp FRAGMENT
298 bp FRAGMENT
ln k
4.00
3.00
2.00
1.00
0.00
−1.00
−2.00
3.05x10⁻³
3.10x10⁻³
3.15x10⁻³
3.20x10⁻³
3.25x10⁻³
3.30x10⁻³
1/T [°K⁻¹]

FIG.—4

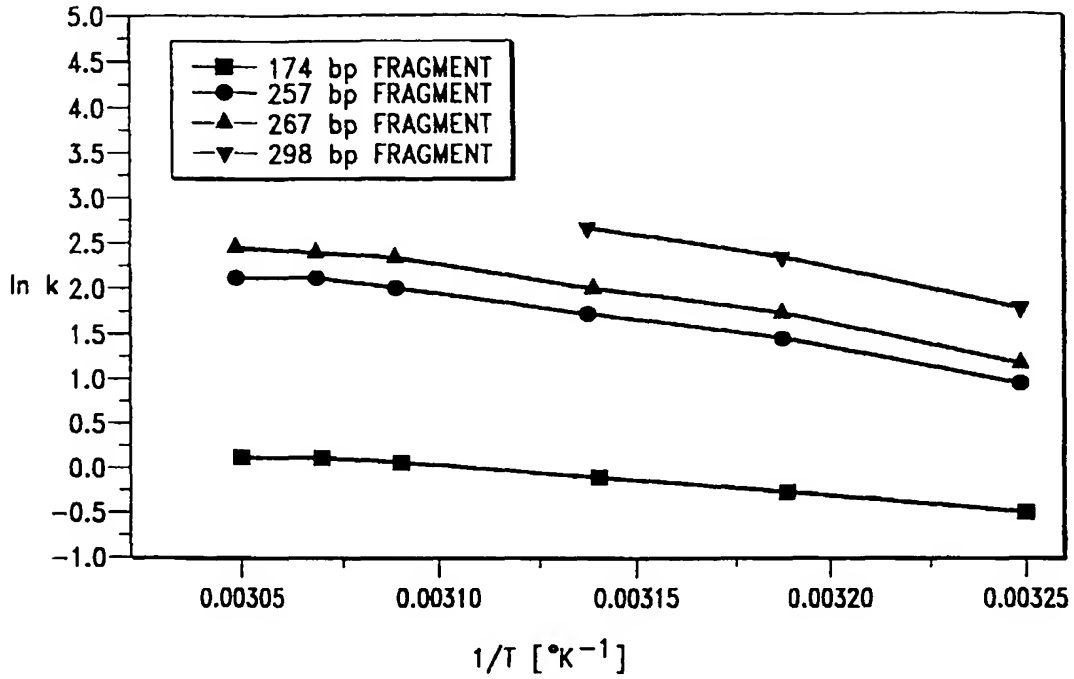

174 bp FRAGMENT
257 bp FRAGMENT
267 bp FRAGMENT
298 bp FRAGMENT
ln k
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
0.00305
0.00310
0.00315
0.00320
0.00325
1/T [°K−1]

FIG.—5

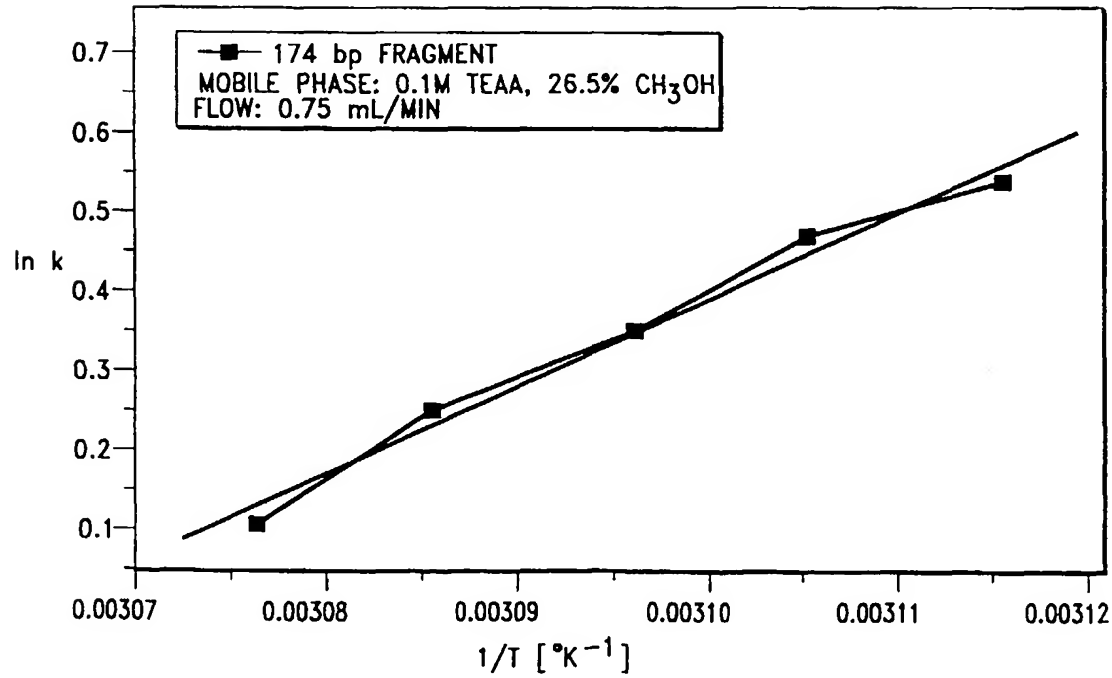

174 bp FRAGMENT
MOBILE PHASE: 0.1M TEAA, 26.5% CH3OH
FLOW: 0.75 mL/MIN
ln k
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.00307
0.00308
0.00309
0.00310
0.00311
0.00312
1/T [°K−1]

FIG.—6

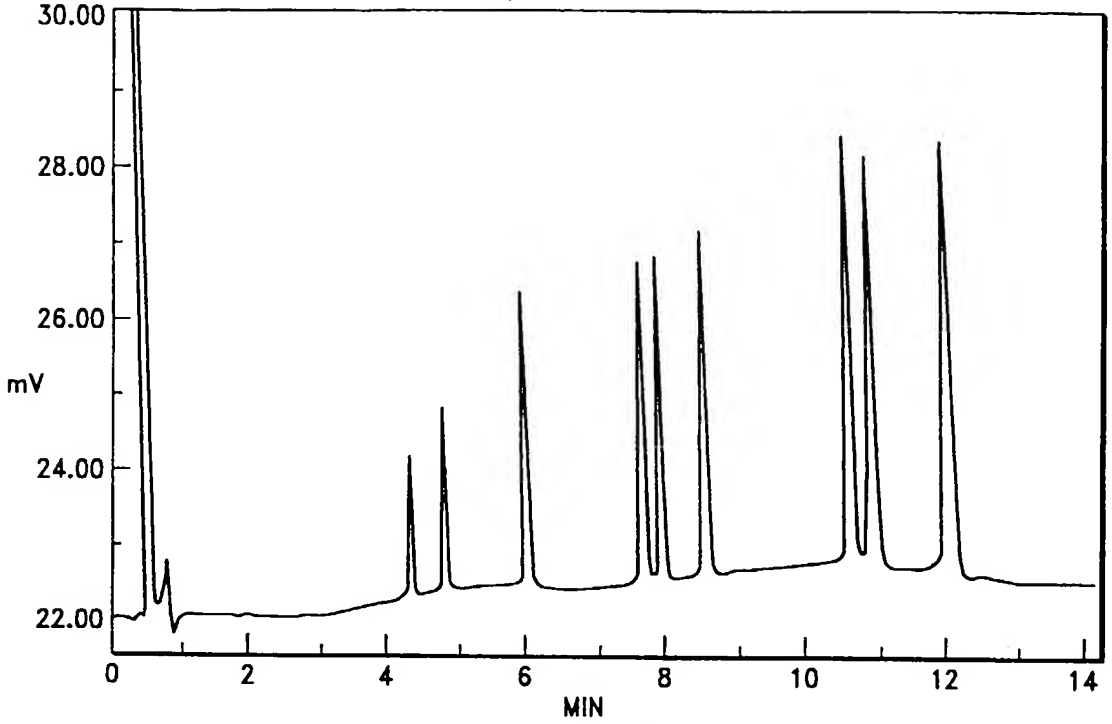

30.00
28.00
26.00
mV
24.00
22.00
0
2
4
6
8
10
12
14
MIN

FIG.—7

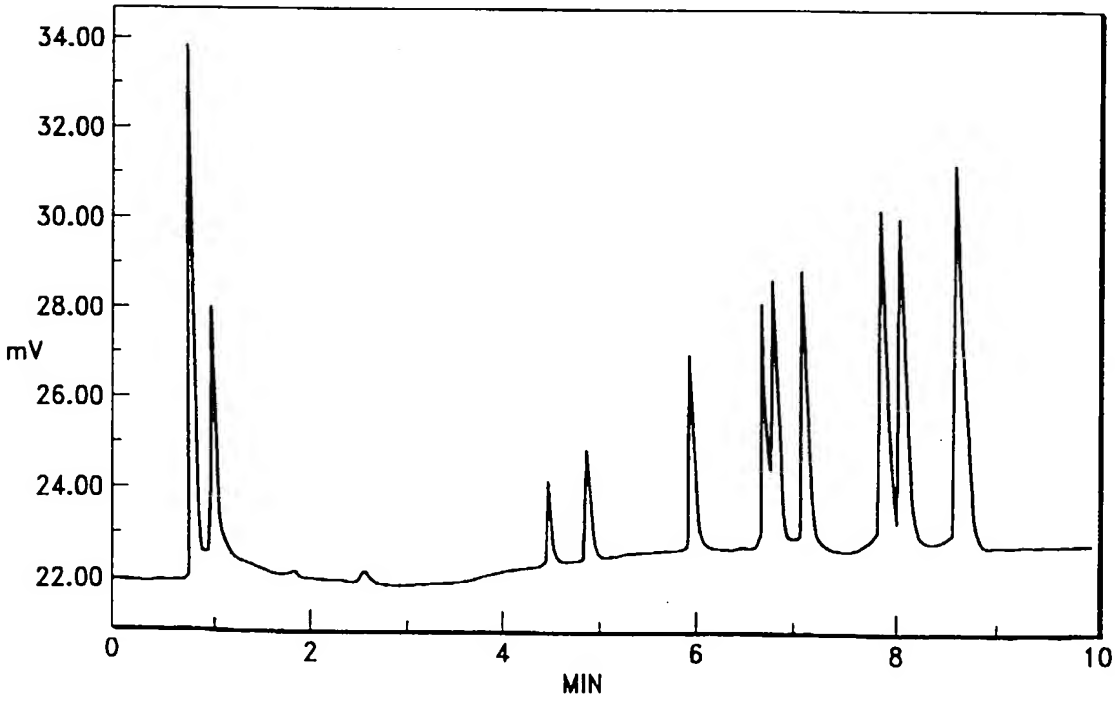

34.00
32.00
30.00
28.00
mV
26.00
24.00
22.00
0
2
4
6
8
10
MIN

FIG.—8

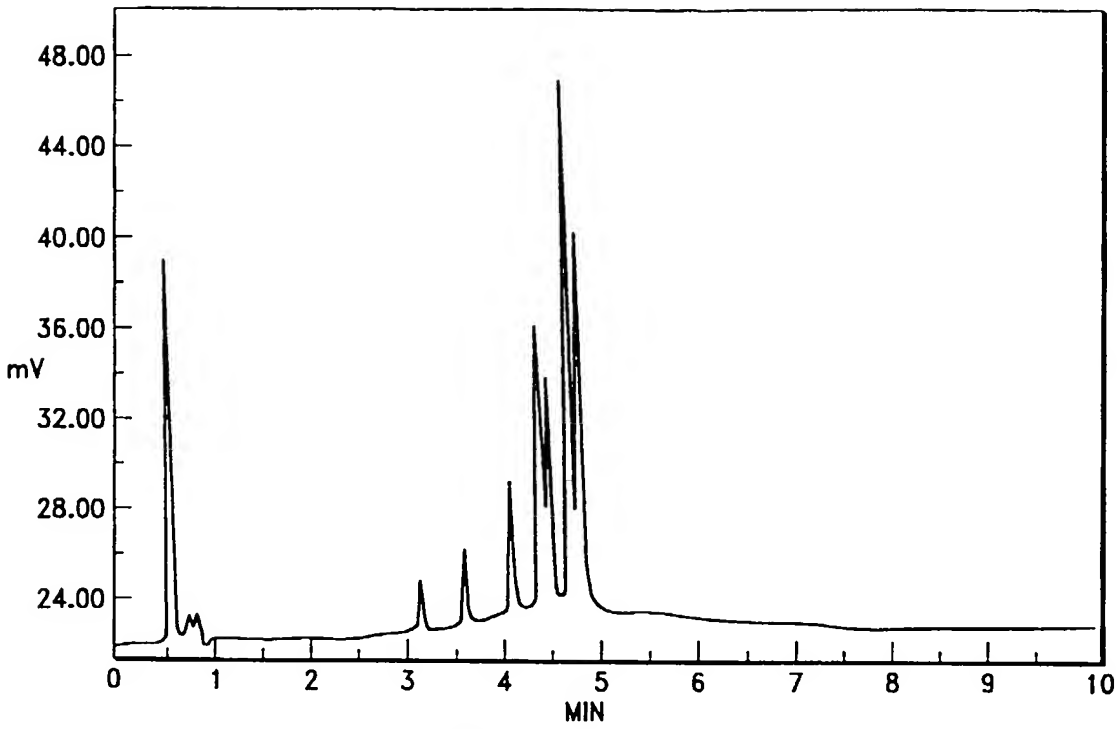

48.00
44.00
40.00
36.00
mV
32.00
28.00
24.00
0
1
2
3
4
5
6
7
8
9
10
MIN

FIG.—9

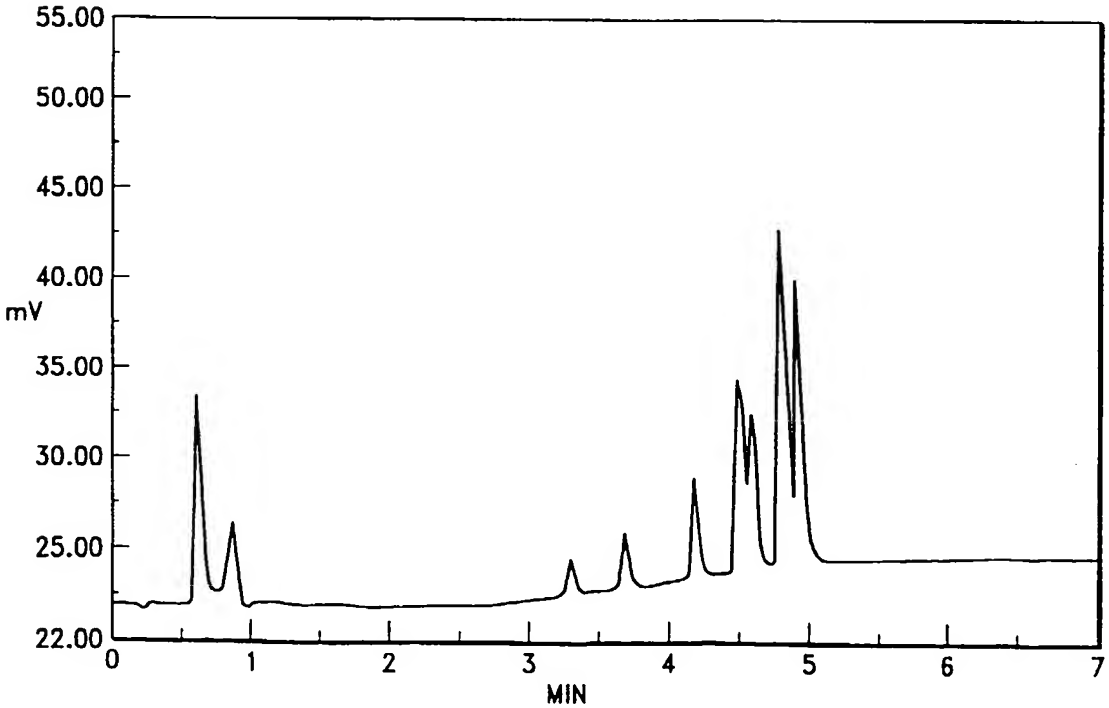

55.00
50.00
45.00
40.00
mV
35.00
30.00
25.00
22.00
0
1
2
3
4
5
6
7
MIN

FIG.—10

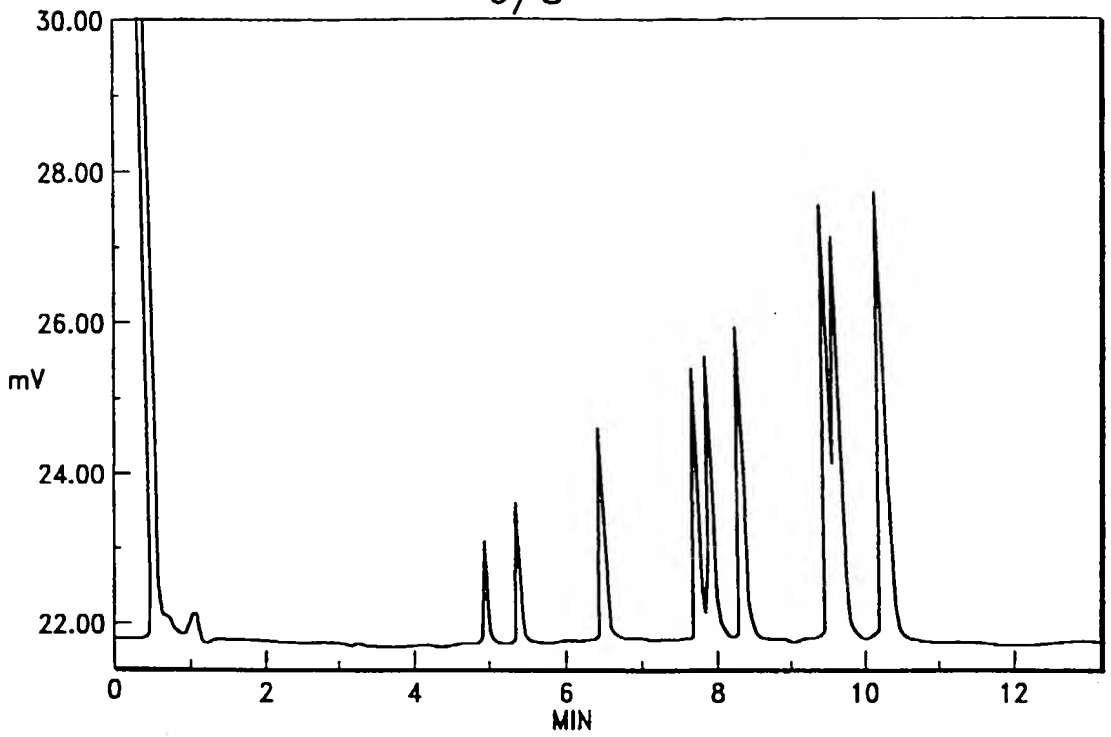

30.00
28.00
26.00
mV
24.00
22.00
0
2
4
6
8
10
12
MIN

FIG.—11

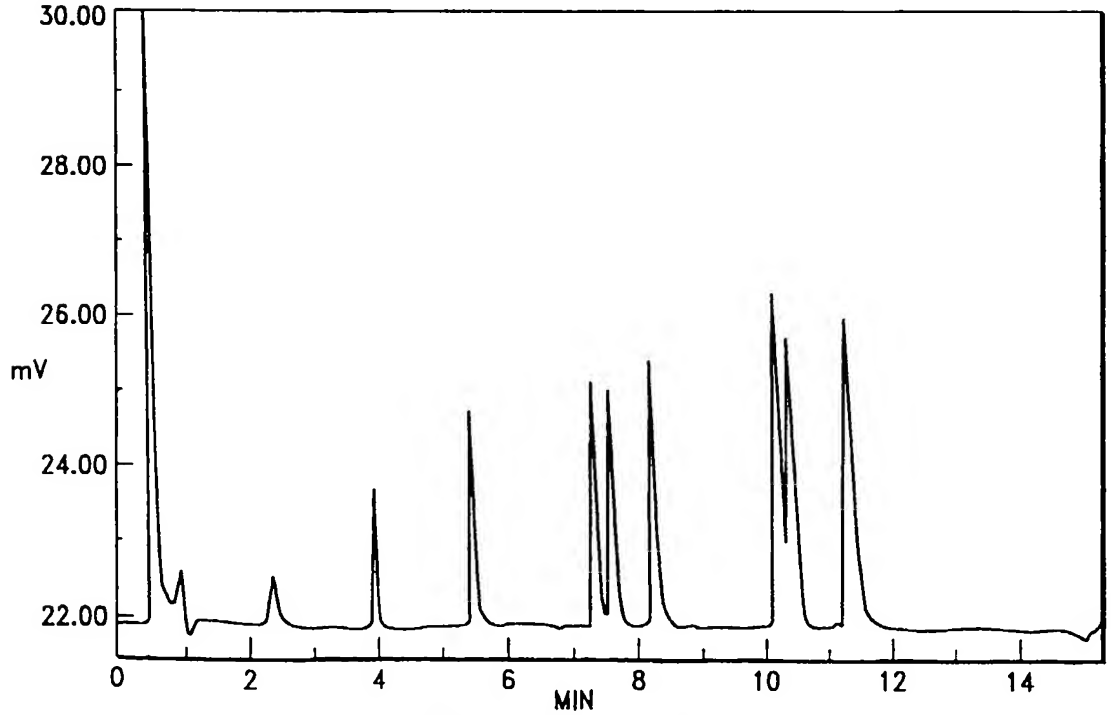

30.00
28.00
26.00
mV
24.00
22.00
0
2
4
6
8
10
12
14
MIN

FIG.—12

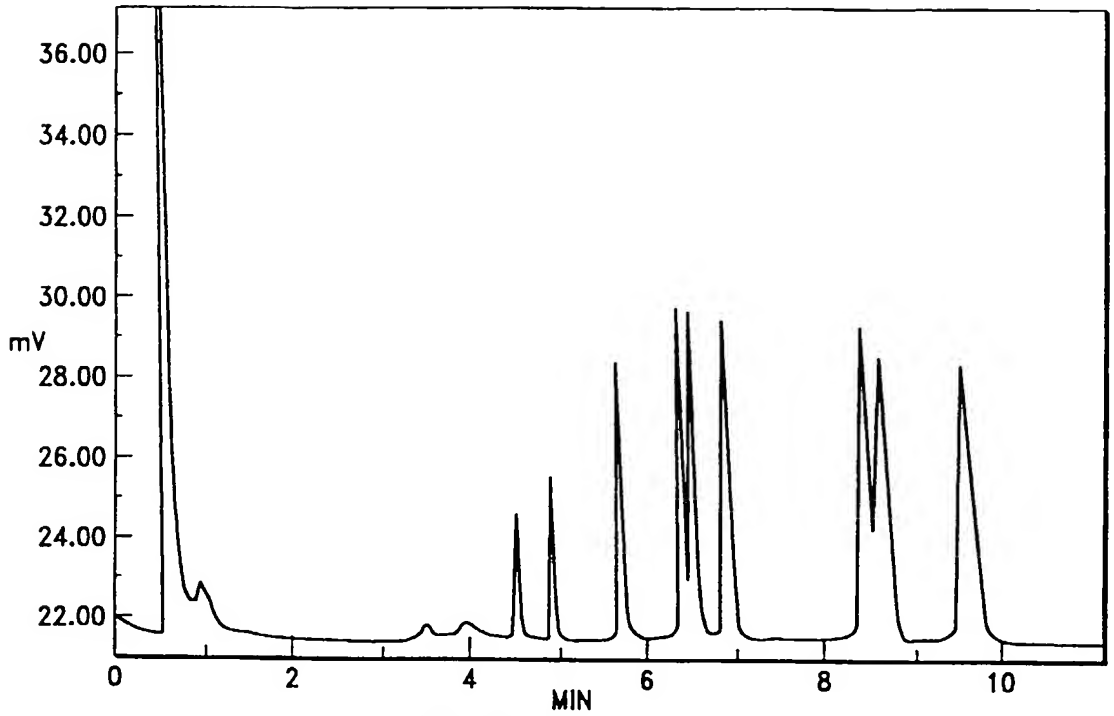

36.00
34.00
32.00
30.00
mV
28.00
26.00
24.00
22.00
0
2
4
6
8
10
MIN

FIG.—13

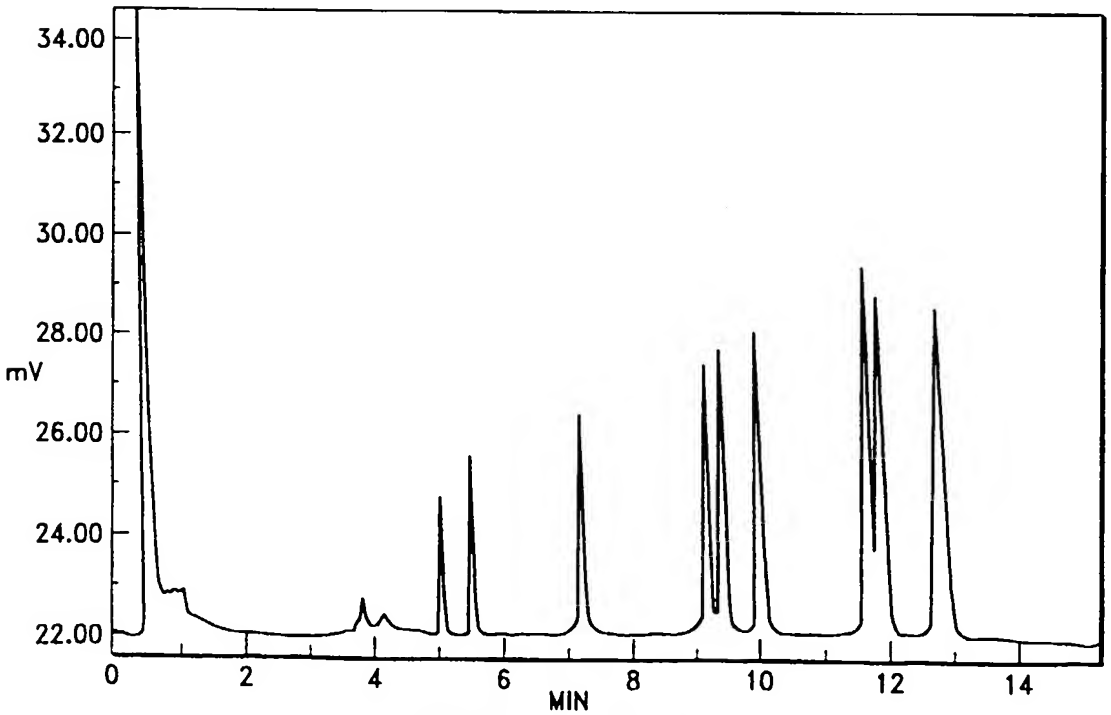

34.00
32.00
30.00
28.00
mV
26.00
24.00
22.00
0
2
4
6
8
10
12
14
MIN

FIG.—14

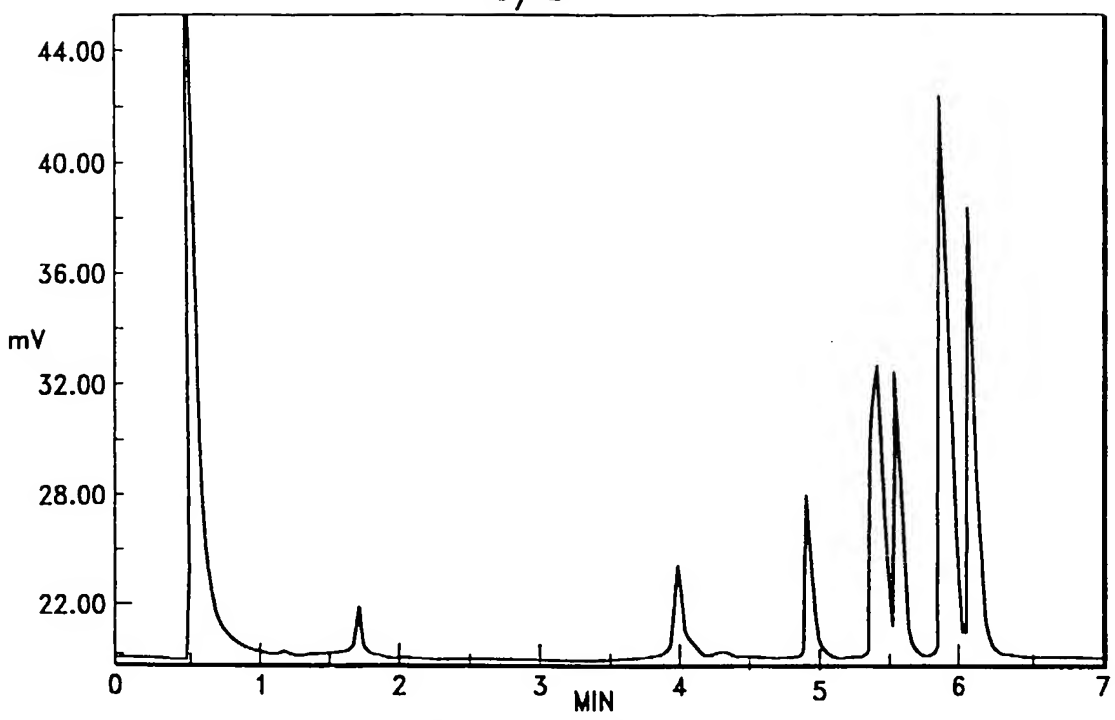

44.00
40.00
36.00
mV
32.00
28.00
22.00
0
1
2
3
MIN
4
5
6
7

FIG.—15

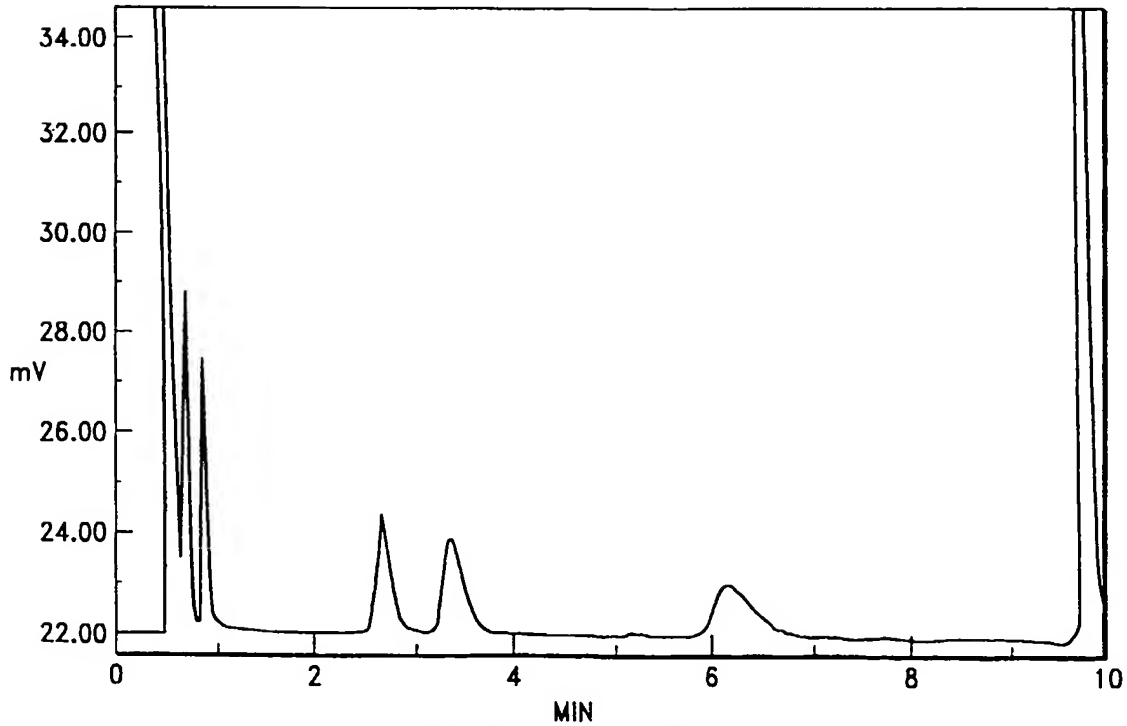

34.00
32.00
30.00
28.00
mV
26.00
24.00
22.00
0
2
4
6
8
10
MIN

FIG.—16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08388

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :B01D 15/00, 15/08; C12Q 1/68; C08F 112/08; C07H 19/00

US CL :210/656, 666; 435/6, 803; 526/347.2; 536/22.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 210/656, 666; 435/6, 803; 526/347.2; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

search terms: polynucleotide, chromatograhy, beads, polymer, separation factor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,585,236 A (BONN et al.) 17 December 1996, see entire document.	1-35
X	HUBER et al. High-Resolution Liquid Chromatography of Oligonucleotides on Nonporous Alkylated Styrene-Divinylbenzene Copolymers. Analytical Biochemistry. 1993. Vol. 212. pages 351-358, see entire document.	1-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 AUGUST 1998	Date of mailing of the international search report 10.09.98
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEZIA RILEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*